

Applicants:	Benvenisty	Atty Docket:	1822/117
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Date Filed:	November 27, 2001	Examiner:	Ton, Thaian N.
Invention:	Transfection of Human Embryonic Stem cells		

I hereby certify that this document, along with any other papers referred to as being attached or enclosed, is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on April 18, 2005.

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In support of the Response filed on April 18, 2005 submitted herewith in reply to the Office Action of October 17, 2004 for the above-referenced matter, I hereby declare as follows:

I. My name is Nissim Benvenisty, M.D., Ph.D. I am a Professor at The Hebrew University in Jerusalem, Israel, in the Department of Genetics. I was formerly the Vice Chair of the Institute of Life Sciences at The Hebrew University, and have been a visiting Professor in the Department of Genetics at Harvard University in Boston, MA, among other positions. I have been awarded numerous prizes and fellowships,

including the Teva Prize for excellent research in stem cells in 2003, the Herbert Cohn Chair in Cancer Research during 1999, the Hestrin Prize in Molecular Biology in 1997, a Howard Hughes Postdoctoral Fellowship from 1991-1993, and a Fulbright Postdoctoral Fellowship from 1990-1991. I have published extensively in the field of stem cell research, and other areas, with over 65 publications, to date. I am also an inventor or co-inventor of a substantial number of patents involving (among other things) human embryonic stem cell research, and I am a co-inventor of the invention claimed in the current application. My further credentials are set forth in my Curriculum Vitae, which is attached hereto as Exhibit A.

2. I have read the action of November 17, 2004. This declaration is provided to clarify the record concerning transfection of human embryonic stem cells relative to transfection of mouse and other animal embryonic stem cells. In particular, I intend a) to provide clear and convincing evidence why it is not obvious to transfect DNA into human ES cells using transfection protocols other than electroporation because the transfection method of choice, until our invention, was electroporation in organisms as varied as bacteria to mice; b) to explain why achieving successful transfection of hES cells using protocols other than electroporation is unexpected and surprising; and c) to explain why those skilled in the art would not expect that combination of the cited prior art, and modifications of mouse and other animal cell transfection protocols found therein, would be successful in achieving efficient transfection of human embryonic stem cells.

Consideration of the Prior Art

3. Since its advent, electroporation has proven to be the method of choice for transfection, from *E. coli* to mammalian systems. Even today, a biotechnology company called Genetronics, Inc. that is developing gene therapy commercially, and thus in need of efficient means for introducing genes into human cells, uses electroporation as the transfection means of choice, and gives a little tutorial on its website of the advantages of electroporation over other standards means of transfection, and recites the disadvantages of those other transfection methods. The Genetronics, Inc. website (<http://www.genetronics.com/sciencegenedelivery.htm>) (see attached Exhibit B) states:

Gene therapy is the treatment of genetic and acquired diseases through the insertion of genes into cells. ... However a gene is a large, chain-like molecule, and difficult to get into a cell. The method frequently being used today is to attach the gene to a disabled virus ...[such as a] retrovirus whose properties have been extensively studied. Retrovirus-mediated transfer *is viewed with some misgivings*, since it brings the gene of the virus into the cells along with the possibility of mutation.

Other methods of gene delivery-such as liposomes, cationic lipids, microinjection or biolistic gun-typically yield *inefficient gene transfer and expression* or are inconvenient, invasive and costly. These methods and viral vectors *can cause immune complications* or limit the size of the DNA being delivered. Electroporation, however, poses few complications and is applicable to a wide variety of targets.

4. Until our successful transfection of hES cells, no other laboratory had succeeded in stable transfecting hES cells. A News Focus article published in Science on March 8, 2002 (vol. 295, pp. 1818 – 1820) credits our laboratory with this achievement (see p. 1819, top of col. 3 – “They struck again ... with the first report on stable genetic

modification of human ES cells. In that work, they inserted into stem cells a gene for green fluorescent protein...”).

5. Moreover, until our successful transfection of hES cells using chemical transfection, those in the field, including my laboratory, expected that electroporation would be the transfection protocol of choice for human ES cells. This was based on the almost universal success seen when transfecting numerous organisms as diverse as bacteria and mice using electroporation, including previous successful transfection of mouse ES cells using electroporation. At the time this invention was filed, and for several years thereafter, the expectation by scientists in the field was that electroporation would be the transfection protocol of choice for human ES cells. The references cited by the Examiner which show successful transfection of mouse ES cells using electroporation merely reinforce this expectation.
6. But electroporation just does not work as well transfecting hES cells as it does in almost every other organism, as we soon discovered, along with others. Even as late as 2003 researchers were reporting lower than expected transfection rates in hES cells when electroporation was used (as seen in a Technical Report by Zwaka and Thomson (*Nature Biotech* (2003), vol. **21**, pp. 319-321– submitted with Declaration 1 as Exhibit C)). Zwaka et al. state that “For human ES cells, the best chemical reagents yield stable (drug-selectable) transfectants at rates of about 10^{-5} ; mouse ES cell electroporation procedures yield substantially lower rates.” The electroporation transfection rate in that article was reported as being only about $\sim 10^{-7}$ when mouse electroporation protocols were adapted and applied for transfection of hES cells (see Zwaka, p. 319, col. 2, first para). In this respect, the entire field was initially taken by

surprise but it soon became clear that electroporation did not introduce DNA into hES at rates or levels high enough to do meaningful research with genetically altered human ES stem cells.

7. Moreover, Figure 1 in the application shows just how inefficient electroporation in hES cells actually is. At first glance, it appears from Fig. 1 that only transfection in the presence of ExGen 500™ results in transfection rates in hES cells an order of magnitude better than those seen using electroporation. In addition, it appears that transfection with other transfection reagents, e.g. LipofectAMINE Plus™ or FuGENE™, resulted in only marginally better or even worse transfection rates than those seen with electroporation.
8. In truth, the values shown in Figure 1 of the application are inaccurate reflections of the actual transfection rates observed when using chemical transfection in the presence of the transfection reagents, as compared to transfection rates using electroporation. That is because my laboratory determined that electroporation unexpectedly killed almost all of the hES cells during the electroporation procedure, possibly because hES cells are much more fragile than other cells and cannot withstand the electroporation procedure. Thus, the transfection rate depicted for electroporation in Fig 1 is misleadingly high since it reflects a rate based on almost ten times as many original cells as the rates depicted for chemical transfection in the presence of the indicated transfection reagents.
9. As pointed out above, Zwaka et al. (*Nature Biotech* (2003), vol. 21, pp. 319-321 - attached as Exhibit C of my October 25, 2004 Declaration 1), concluded the same thing as late as 2003 – nearly four years after we reported the first successful stable

transfection of human ES cells. As set forth in Zwaka et al., "For human ES cells, the best chemical reagents yield stable ... transfectants at rates of about 10^{-5} ; mouse ES cell electroporation procedures yield substantially lower rates.⁶" (see p. 319, col. 1, 2nd para.). The reference cited (reference 6) is Eiges et al. (see above) a reference for which I am the corresponding author, and the paper which contains much of the core experimental data that forms the basis for this invention. The 2001 Eiges et al. publication, based on research carried out in my laboratory in 2000 and before, is the first publication reporting the transfection of human ES cells.

10. Eiges et al. and Zwaka et al. provide substantial evidence that mouse protocols for introducing DNA into human ES cells using electroporation did not work at the time this invention was submitted and as late as 2003. And considering that our claimed transfection methods require transfection in the presence of at least one cationic lipid, non-lipid cationic, or transfection reagent, those skilled in the art would understand this to mean chemical transfection and not transfection by electroporation.

11. Electroporation may be successful for transfecting murine ES cells, but not for transfecting human ES cells. Because of the substantial differences which exist between mouse and human embryonic stem cells - see the Table in Declaration 1 discussions of same in Kaufman et al. (*Proc. Natl. Acad. Sci. USA*, Vol. 98, No. 19, pp. 10716 - 10721, (2001) - attached in Declaration 1 as Exhibit D) - we were not able to use electroporation to transfect hES cells. Unexpectedly, electroporation killed most of the hES cells during the transfection procedure itself. Therefore, my laboratory started looking for a different transfection methodology for hES cells, which is what ultimately led us to the claimed invention. The fact that we had to look

for alternatives to electroporation prior to 2001, and that Zwaka et al. reiterated in 2003 that transfection using electroporation protocols as performed with mouse ES cells did not work for introducing DNA into hES cells, is more evidence that the devising of a transfection protocol for hES cells was not obvious.

12. The work in our laboratory, as published in Eiges et al. (see exhibit B, Declaration 1) shows that chemical transfection using the transfection reagent ExGen 500™, a polycationic non-lipid polymer, resulted in transfection rates an order of magnitude better than achieved using electroporation, LipofectAMINE Plus™ or FuGENE™ (see Eiges, Figure 1) and that the quantitative transfection rate we achieved was $\sim 10^{-5}$ compared to $\sim 10^{-7}$ for electroporation (see above). What is not apparent from Figure 1 is that we had to start with about 10x as many cells for the electroporation data to measure the rates we observed for transfection with electroporation. Thus, our transfection methods using cationic polymers yielded transfection rates more than an order of magnitude better than electroporation in any system, and that “stable clones were derived in an efficiency of $\sim 10^{-5}$ from the transfected cells” (see Eiges, p. 515, col. 2, last para. and as quoted in Zwaka et al., col. 1, second para.).
13. With respect to transfection reagents and electroporation, those in the field would not perform electroporation in the presence of transfection reagents. A protocol that requires transfection in the presence of transfection reagents, as claimed in our invention, is understood to be a chemical method of transfection. Electroporation is a mechanical means for transfection, not a chemical means, and does not require additional transfection agents to facilitate the entry of nucleic acids into the cell

because the applied electric current acts to disrupt the cell membrane and allow influx of nucleic acids into the cell, so no added transfection reagents are needed.

14. Similarly, those in the field trying to transfect hES cells would not use adenovirus.

Adenovirus transfects cells by infecting them, and infecting hES cells with a virus is potentially too dangerous because of the possibility of introducing or activating oncogenes for it to be considered as a viable means for transfection in hES cells. In short, adenovirus infection is not compatible with the goal of obtaining stably-transfected hES cells for later therapeutic use.

15. In summary, although researchers in the field of human ES cell research may have tried to solve the problem of translating mice and monkey transfection and electroporation protocols to human ES cells efficiently to develop human ES cell lines with altered gene expression, no one was successful until my laboratory showed the way, specifically because it was *not* obvious to achieve our results based on what was already known about transfecting mouse ES cells and other animal cells.

16. I hereby declare that all statements made herein are of my own knowledge and that all statements made on information and belief are true; and further that these statements are being made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Benvenisty, M.D., Ph.D.

Dated: April ___, 2005

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Exhibits:

A - *Curriculum Vitae* of Nissim Benvenisty, M.D., Ph.D.

B – Genetronics, Inc. Webpage, “Electroporation” retrieved from the internet on

4/14/05, url <http://www.genetronics.com/sciencegenedelivery.htm>, Genetronics, Inc.

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NISSIM BENVENISTY

CURRICULUM VITAE

Date and Place of Birth : 9.10.1958; Israel.

Marital Status: Married + 3.

Education :

- 1983 M.D. Faculty of Medicine, Hebrew University.
- 1986 Ph.D. Department of Developmental Biochemistry,
Hadassah Medical School, Hebrew University.

Employment and Related Training :

- 2002- Professor, Department of Genetics,
The Hebrew University, Jerusalem, Israel.
- 2002-2003 Head of Biology Teaching and
Vice Chair, Institute of Life Sciences
The Hebrew University, Jerusalem, Israel
- 1999-2000 Visiting Professor, Department of Genetics,
Harvard University, Boston, USA
- 1998-2002 Associate Professor, Department of Genetics,
The Hebrew University, Jerusalem, Israel.
- 1993-1998 Senior Lecturer, Department of Genetics, The Hebrew
University, Jerusalem, Israel.
- 1990-1993 Research Fellow, Department of Genetics,
Harvard Medical School, Boston, USA.
Under supervision of Professor Philip Leder.
- 1986-1990 Israeli Army Medical Service.
- 1983-1986 Graduate Student, Department of Developmental
Biochemistry, The Hebrew University, Jerusalem, Israel.
- 1985 Research Associate, Case Western Reserve University,
Cleveland, USA.

1983-1984 Internship, Hadassah Hospital, Jerusalem, Israel.

1982-1985 Teaching biochemistry and molecular biology to medical students at The Hebrew University.

Awards and Fellowships :

1981	Awarded the Faculty Prize.
1982	Awarded a Fellowship at the Mount Sinai Hospital, New York - Program for outstanding students.
1982-1985	Foulkes Foundation Fellowship.
1985	Best Teacher Award for teaching biochemistry and molecular biology.
1988	Awarded the Senta Foulkes Prize (London).
1990-1991	Awarded the Weizmann Postdoctoral Fellowship.
1990-1991	Awarded the Fulbright Postdoctoral Fellowship.
1991-1993	Awarded the Howard Hughes Postdoctoral Fellowship.
1993-1996	Awarded the Alon Fellowship.
1994	The Joseph H. and Belle R. Braun Senior Lectureship in Life Sciences.
1995-1998	Awarded Best Teacher in Genetics.
1995	Awarded the Hebrew University Prize for Young Scientist.
1996	Awarded the Naftali Prize.
1997	Awarded the Hestrin Prize in Biochemistry and Molecular Biology.
1998	Awarded the Rom Prize in Genetics
1999	The Herbert Cohn Chair in Cancer Research
1999-2000	Awarded the Yamagiwa-Yoshida Memorial International Cancer Study Fellowship.
2003	Awarded the Teva Prize for excellent research in stem cells

List of publications

1. **Benvenisty, N., Ben-Simchon, E., Cohen, H. Mencher, D., Meyuhas, O. and Reshef, L. :** Control of the activity of phosphoenolpyruvate carboxykinase and the level of its mRNA in livers of newborn rats. Eur. J. Biochem. 132: 663-668 (1983).
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7. **Benvenisty, N., Razin, A. and Reshef, L. :** Developmental changes in the methylation pattern, chromatin conformation and expression of the rat phosphoenolpyruvate carboxykinase gene. In : Biochemistry and Biology of DNA Methylation, G.L. Cantoni and A. Razin, eds. , Alan R. Liss, Inc., New York, pp. 185-199 (1985).
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